

## PEER REVIEW HISTORY

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### ARTICLE DETAILS

<b>TITLE (PROVISIONAL)</b>	Standardised Treatment And Monitoring Protocol to assess safety and tolerability of bacteriophage therapy for adult and paediatric patients (STAMP study): protocol for an open-label, single-arm trial
<b>AUTHORS</b>	Khatami, Ameneh; Foley, David; Warner, Morgyn; Barnes, Elizabeth; Peleg, Anton; Li, Jian; Stick, Stephen; Burke, Nettie; Lin, Ruby; Warning, Julia; Snelling, Thomas; Tong, Steven; Iredell, Jonathan

### VERSION 1 – REVIEW

<b>REVIEWER</b>	Rémy Froissart Centre National de la Recherche Scientifique (CNRS), MIVEGEC
<b>REVIEW RETURNED</b>	27-Jul-2022

<b>GENERAL COMMENTS</b>	<p>The purpose of this study is to standardise therapeutic management and data collection for assessing the overall safety and tolerability of phage therapy in children and adults, and to assess the feasibility of a standardised national protocol.</p> <p>I first have to thank the authors for their effort of adresssing this purpose and I agree with them that publication of such protocols is absolutely needed for the phage-therapy community to homogenize their way of accumulating data.</p> <p>However, I was a bit surprised with the text that is difficult to follow because of repetitions of 2 very similar (but not identical) text (page 121 to 165 versus 1 to 68) with the middle part (69 to 120) that is not understandable...</p> <p>Disappointingly, the aim of the authors to provide a protocol that would be repeatable was not acheived. See below all the details that are lacking for anyone to repeat what is claimed to be done during the trial ACTRN12621001526864. However, I'm sure that the authors will be able to respond to all my demands.</p> <p>%%%%%%%%%</p> <p>Page 9 Line 27-29 : "Biofilms protect bacteria from the immune response and reduce antibiotic efficacy" Biofilm has other multiple functions - the way it is presented is too restrictive. Moreover, not all phages present depolymerases able to destroy biofilm... so, as it is presented is not exact.</p> <p>Page 9 Line 37 : what does authors mean by: "sufficiently purified phages are generally not readily available". What is the level of purification acceptable ?</p>
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	<p>Page 10 line 22-23 : "As PT experience grows this may include external partners." Please, be more precise : partners with which background ?</p> <p>Page 10 line 28 : "A phage product must demonstrate high in vitro activity against the targeted pathogen(s)" -- what authors mean by "high activity".</p> <p>page 10 line 30-31 : could authors refer to any monography for phage production and/or pharmacopia protocols for purification of phages ?</p> <p>Page 10 and further -- Methods:</p> <ol style="list-style-type: none"> <li>1- no paragraph describe the way etiologic bacteria are sampled nor the number of colonies tested against the collection of phages. Also describe the way doctors establish the link between symptoms and the etiologic bacteria. This information is essential to target the good pathogen (see Brussow T4/EHEC failure because the symptoms were not caused by EHEC but Streptococcus)</li> <li>2- no description is made of the phage collection the bacteria will be tested against</li> <li>3- no description is made of the phagogramme protocol</li> </ol> <p>Page 13 Line 31 (and page 14 line 42): "Phage antibodies" quantification: please describe the kind of antibodies that will be followed (IgG, IgA, IgM, etc) and following which method. Moreover, is it antibodies in a whole or neutralizing antibodies that will be quantified ? if so, explain how (see page 138 line 12-20)</p> <p>Page 13 line 50: "10 9 plaque-forming units (pfu) of phage at each dose" / "For non-intravenous routes of administration, approximately 10 10 pfu/dose will be administered": please give the total volume of each dose</p> <p>Page 14 line 2: "Phage quantification in blood will be determined by plaque assay and quantitative polymerase chain reaction (qPCR)." Please give some details on how DNA will be extracted and how internal control of qPCR will be handle (which target of phages and blood cells). Which quantity of blood will be sampled ? Also, will the concentration of bacteria quantified before and after phage applications ? if yes, explain how (qPCR, CFU/mL) and where (infection site and/or blood). This point might be addressed on page 16 in paragraph "Microbiological Clearance": please indicate how the bacterial cultures will be done (liquid ? solid cultures ? selective medium ? antibiotic susceptibility level ?)</p> <p>Page 16 line 48: please indicate how will be done the "Metagenomics": 16S or shotgun ? how to distinguish virulent to "endogenous" (line 52) phages ? How to determine if the "endogenous" phages are induced or not ?</p> <p>Document was not understandable from page 69 to 121, presumably due to a bug in the submission file set-up. Page 121 to 165 correspond to a text very similar to the one presented from 1 to 68 but some usefull information are present in the second part. For instance, paragraphe "11.3.2 Microbiology" (page 134 line 13-47) concerning the origin of the phages and the fact that they were sequenced, is an important information. Please homogenize !</p> <p>Also, several comments:</p> <ol style="list-style-type: none"> <li>1- line 20, please describe who will provide the bacteria allowing the</li> </ol>
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	<p>production of phage stock and give information on the safety checking of these production-bacteria (absence of prophage, absence of antibiotic resistance genes (ARG), absence of toxins), etc.: provide the bio-informatic softwares that will be used</p> <p>2- line 33 "Whole genome sequencing (WGS) of main pathogen(s) according to laboratory standard operating procedures (SOP), and identification of prophages": please consider also checking for presence of ARG and toxins: provide the bio-informatic softwares that will be used</p> <p>3- line 38 "Phage-bacteria-antibiotic synergy testing according to laboratory SOP." please provide a reference for the protocol that will be used.</p> <p>Page 135 line 1: give the followed protocol to estimate the level of endotoxins for both Gram negative and positive bacteria.</p> <p>Page 138 line 54: since susceptibility of bacteria to phages will be tested, please precise on how many colonies will be tested "All positive cultures"</p> <p>Page 138 line 60: "Transcriptomics and metagenomics" : what does it means ? what will be do and how ?</p> <p>Page 140 line 41: "Bacterial cultures should be obtained from the site of infection" -- same as before: how many colonies will be tested, how to estimate the genotypical diversity at each site?</p>
<b>REVIEWER</b>	Patrick Jault Clinique de la Muette
<b>REVIEW RETURNED</b>	23-Oct-2022
<b>GENERAL COMMENTS</b>	<p>Well designed protocol.</p> <p>The first clinical trial previously published on the clinical of phages is not cited in your references (JAULT and Coll , 2019, The Lancet infectious diseases)</p>

### VERSION 1 – AUTHOR RESPONSE

Reviewer: 1 Dr. Rémy Froissart, Centre National de la Recherche Scientifique (CNRS)

Comments to the Author:

The purpose of this study is to standardise therapeutic management and data collection for assessing the overall safety and tolerability of phage therapy in children and adults, and to assess the feasibility of a standardised national protocol. I first have to thank the authors for their effort of addressing this purpose and I agree with them that publication of such protocols is absolutely needed for the phage-therapy community to homogenize their way of accumulating data.

Response: We thank Dr. Froissart for his kind comments.

However, I was a bit surprised with the text that is difficult to follow because of repetitions of 2 very similar (but not identical) text (page 121 to 165 versus 1 to 68) with the middle part (69 to 120) that is not understandable...

Response: We apologise for the confusion. Pages 121 to 165 is the full trial protocol as approved by HREC and included as supplementary material to the manuscript. Pages 1 to 68 is the manuscript version of the protocol, formatted to the journal style. Page 69 to 120 is the REDCap data registry data dictionary which must be included but does not format correctly as a pdf.

Disappointingly, the aim of the authors to provide a protocol that would be repeatable was not achieved. See below all the details that are lacking for anyone to repeat what is claimed to be done

during the trial ACTRN12621001526864. However, I'm sure that the authors will be able to respond to all my demands.

Response: We thank Dr. Froissart for his helpful comments, which are addressed below.

Page 9 Line 27-29 : "Biofilms protect bacteria from the immune response and reduce antibiotic efficacy"

Biofilm has other multiple functions - the way it is presented is too restrictive. Moreover, not all phages present depolymerases able to destroy biofilm... so, as it is presented is not exact.

Response: Thank you for highlighting this. This point regarding biofilms is specifically related to their role in "difficult-to-treat" human infections, rather than, for example, protecting bacteria from environmental stresses. We have made a few minor edits to help clarify this.

Page 9 Line 37 : what does authors mean by: "sufficiently purified phages are generally not readily available". What is the level of purification acceptable ?

Response: We have clarified that the level of purification that is often sought for therapeutic products is production in a licensed Good Manufacturing Process (GMP) facility. Outside of that, there is no specific level of purification that is acceptable across all jurisdictions or health care facilities beyond a general specification that endotoxin not be administered in excess of the pyrogenic threshold of 5 EU/kg/hour. This is part of the challenge regarding regulation of therapeutic phages, however more detailed discussion around this point is beyond the scope of this manuscript.

Page 10 line 22-23 : "As PT experience grows this may include external partners." Please, be more precise : partners with which background ?

Response: This sentence has been deleted to avoid ambiguity.

Page 10 line 28 : "A phage product must demonstrate high in vitro activity against the targeted pathogen(s)" -- what authors mean by "high activity".

Response: Thank you for this comment. We have now added explanatory text and relevant reference. In general, the activity purity and safety of the preparation must satisfy the responsible authorities, including the institutions and individuals (physicians) with a direct duty of care and all relevant regulatory bodies where specific guidelines exist (see also response to comments below).

page 10 line 30-31 : could authors refer to any monography for phage production and/or pharmacopia protocols for purification of phages ?

Response: Thank you for this comment, however we feel that this is beyond the scope of the manuscript. The aim of this protocol is to standardise the administration and monitoring of phage therapy, i.e., the "process" of phage therapy. The actual phage products being administered are not under direct scrutiny and phage production and purification are explicitly outside of the scope of the trial protocol. Eligibility criteria include "that a suitable phage product that meets all regulatory requirements and local approvals has been identified". Many different types of phages could be used including wild-type or engineered, and many different levels of engineering could be utilised including ligated chemicals etc. Phages could be sourced locally or internationally, from commercial or academic institutes, etc. As such, we would not be able to reference a particular protocol for production/purification. However, we have now added supplementary material as examples of quality control documentation that could be provided to relevant authorities (e.g., institutional drug committee providing approval for therapy) from phage suppliers (supplement B, mentioned in the Participant Recruitment and Eligibility section). These examples are from Phage Australia, for locally produced phage products; however, as noted above, therapeutic phage products used in this trial could be sourced externally, including from international sources or commercial suppliers.

Page 10 and further -- Methods:

1- no paragraph describe the way etiologic bacteria are sampled nor the number of colonies tested against the collection of phages. Also describe the way doctors establish the link between symptoms and the etiologic bacteria. This information is essential to target the good pathogen (see Brussow T4/EHEC failure because the symptoms were not caused by EHEC but Streptococcus)

2- no description is made of the phage collection the bacteria will be tested against

3- no description is made of the phagogramme protocol

Response: Thank you for these comments, however we feel that some of these questions are beyond the scope of the manuscript. The aim of this protocol is to standardise the administration and monitoring of phage therapy, once that therapy has been approved by the relevant authorities. The actual phage products being administered are not under direct scrutiny, which is why "efficacy" (microbiological or clinical) have only been included as exploratory outcomes for the trial. The pragmatic nature of the protocol ensures that it is easily embedded in routine clinical practice (to improve participant recruitment/retention). As such, many "interventions" such as microbiological sampling are in part determined by the treating clinicians. Furthermore, eligibility criteria for enrolment in the trial include "that a suitable phage product that meets all regulatory requirements and local approvals has been identified". As such, bacterial sampling and submission for phage susceptibility testing would occur prior to and outside of the trial protocol. Table 1 of the full trial protocol (submitted as a supplement to the manuscript) highlights activities that are clinical activities, versus those that are research activities. Since many different types of phages could be used, sourced locally or internationally, from commercial or academic institutes, etc., we have not made a specific reference to the phage collections to be tested against, or the protocols for such.

We had, however, specified that "Eligibility will be limited to patients assessed as having exhausted other therapeutic options to control their infection. This assessment will be performed by two appropriately qualified clinical specialists and must include a specialist in infection management and a specialist with prior PT experience..." We have now added "... where the clinical syndrome is linked directly to the aetiologic bacteria targeted" to highlight the important point raised by Dr Friossart.

Page 13 Line 31 (and page 14 line 42): "Phage antibodies" quantification: please describe the kind of antibodies that will be followed (IgG, IgA, IgM, etc) and following which method. Moreover, is it antibodies in a whole or neutralizing antibodies that will be quantified ? if so, explain how (see page 138 line 12-20)

Response: Antibody measurements will vary according to phage product used, site of infection, route of administration and laboratory performing the assay. These are not routinely available assays, nor are they validated tests. Since these are exploratory outcomes for the trial, specifying particular assays to be performed would only limit participant recruitment by creating challenges for participating sites. We have now referenced one such example for one laboratory that performs neutralising antibody assays, however many other assays may be used.

Page 13 line 50: "10<sup>9</sup> plaque-forming units (pfu) of phage at each dose" / "For non-intravenous routes of administration, approximately 10<sup>10</sup> pfu/dose will be administered": please give the total volume of each dose

Response: The total volume to be administered will vary according the phage product used including phage titre and endotoxin content, weight of the patient and route of administration.

Page 14 line 2: "Phage quantification in blood will be determined by plaque assay and quantitative polymerase chain reaction (qPCR)." Please give some details on how DNA will be extracted and how internal control of qPCR will be handle (which target of phages and blood cells). Which quantity of blood will be sampled?

Response: The laboratory assays to be used for monitoring are not specified in the trial protocol, as they will vary, depending on the phage product and the laboratory performing the assay (which may be one of many across Australia). It is expected that such monitoring assays in most countries are only performed in laboratories subject to regular accreditation by a national regulatory authority which

ensures compliance with ISO15189 (Requirements for Quality Management in Medical Laboratories). More discussion of this issue is now included in the "Laboratory Monitoring" section of the manuscript.

Also, will the concentration of bacteria quantified before and after phage applications ? if yes, explain how (qPCR, CFU/mL) and where (infection site and/or blood). This point might be addressed on page 16 in paragraph "Microbiological Clearance": please indicate how the bacterial cultures will be done (liquid ? solid cultures ? selective medium ? antibiotic susceptibility level ?)

Response: Where possible, bacterial target populations will be quantified by qPCR as genome copies/mL in whole blood. This had been mentioned in Table 4 of the manuscript but specific reference to this is now made in the "Laboratory Monitoring" section. Thanking for pointing out this oversight. The references given describe assays in routine use in our own laboratories.

Microbiological clearance will be determined as per routine clinical practice depending on the participating site where patients are being treated. These cultures will be performed by nationally accredited diagnostic laboratories according to their own SOPs. Methods will vary according to the bacterial target.

Page 16 line 48: please indicate how will be done the "Metagenomics": 16S or shotgun ? how to distinguish virulent to "endogenous" (line 52) phages ? How to determine if the "endogenous" phages are induced or not?

Response: See also response to comment/query above. The laboratory assays to be used for these monitoring assays are not specifically included in the trial protocol.

Document was not understandable from page 69 to 121, presumably due to a bug in the submission file set-up. Page 121 to 165 correspond to a text very similar to the one presented from 1 to 68 but some useful information are present in the second part. For instance, paragraph "11.3.2 Microbiology" (page 134 line 13-47) concerning the origin of the phages and the fact that they were sequenced, is an important information. Please homogenize!

Response: See also response to comment above. We apologise for the confusion. Pages 121 to 165 is the full trial protocol as approved by HREC and included as supplementary material to the manuscript. Pages 1 to 68 is the manuscript version of the protocol, formatted to the journal style. Page 69 to 120 is the REDCap data registry data dictionary which must be included but does not format correctly as a pdf.

Also, several comments:

1- line 20, please describe who will provide the bacteria allowing the production of phage stock and give information on the safety checking of these production-bacteria (absence of prophage, absence of antibiotic resistance genes (ARG), absence of toxins), etc.: provide the bio-informatic softwares that will be used

Response: Thank you for this comment, however phage production and purification are explicitly outside of the scope of the trial protocol (see also response to comments above). Eligibility criteria include "that a suitable phage product that meets all regulatory requirements and local approvals has been identified". The trial (which aims to standardise the administration and monitoring of phage therapy) starts after a phage product has already been selected for therapeutic use. The actual phage products being administered are not under direct scrutiny. Many different types of phages could be used including wild-type or engineered, and phages could be sourced locally or internationally, from commercial or academic institutes, etc. However, we have now added supplementary material as examples of quality control documentation that could be provided to relevant authorities (e.g., hospital drug committee providing approval for therapy) from phage suppliers (supplement B). These examples are from Phage Australia, for locally produced phage products, however, as noted, therapeutic phage products could be sourced externally, including from international sources or commercial suppliers.

2- line 33 "Whole genome sequencing (WGS) of main pathogen(s) according to laboratory standard operating procedures (SOP), and identification of prophages": please consider also checking for presence of ARG and toxins: provide the bio-informatic softwares that will be used

Response: Please see response to similar comments above. The laboratory assays to be used for monitoring are not specified in the trial protocol, as they will vary, depending on the phage product and the laboratory performing the assay (which may be one of many across Australia). It is expected that such monitoring assays in most countries are only performed in laboratories subject to regular accreditation by a national regulatory authority which ensures compliance with ISO15189 (Requirements for Quality Management in Medical Laboratories). More discussion of this issue is now included in the "Laboratory Monitoring" section of the manuscript.

3- line 38 "Phage-bacteria-antibiotic synergy testing according to laboratory SOP." please provide a reference for the protocol that be used.

Response: This is not mentioned in the manuscript but is listed in the protocol. We assume it is page 134 that Dr Froissart is referring to here. Phage-bacteria-antibiotic synergy testing is not an essential assay for the protocol which is why it was not specifically mentioned in the manuscript. As you will see in the full trial protocol, some tests are highlighted with an \* as Minimum Data Requirements. Synergy assays are not included in this list as these are very difficult to perform and interpret. In our lab, we use a standard method similar to the one used by Ronen Hazan, see Gelman et. al., Lancet Microbe 2021; 2: e555–63.

Page 135 line 1: give the followed protocol to estimate the level of endotoxins for both Gram negative and positive bacteria.

Response: Thank you for this comment, however phage production and purification are explicitly outside of the scope of the trial protocol (see also response to comments above). Eligibility criteria include "that a suitable phage product that meets all regulatory requirements and local approvals has been identified". The trial (which aims to standardise the administration and monitoring of phage therapy) starts after a phage product has already been selected for therapeutic use. This would include information from the supplier of the phage product (phages could be sourced locally or internationally, from commercial or academic institutes, etc.) regarding endotoxin content and other relevant quality control measures (bacterial contamination etc.). However, we have now added supplementary material as examples of quality control documentation that could be provided to relevant authorities (e.g., hospital drug committee providing approval for therapy) from phage suppliers (supplement B). These examples are from Phage Australia, for locally produced phage products, however, as noted above, therapeutic phage products could be sourced externally, including from international sources or commercial suppliers.

Page 138 line 54: since susceptibility of bacteria to phages will be tested, please precise on how many colonies will be tested "All positive cultures"

Response: Thank you for these comments, however the protocol has been specifically designed to be pragmatic and easily embedded in routine clinical practice (to improve participant recruitment/retention). As such, many "interventions" such as microbiological sampling are in part determined by the treating clinicians and by standard clinical and microbiological practice of the recruitment site. Phage susceptibility testing will be performed on all positive cultures obtained after initiation of phage therapy, however the number of colonies sampled or sub-cultured and submitted for susceptibility testing will be determined by standard microbiological practice of the recruitment site.

Page 138 line 60: "Transcriptomics and metagenomics" : what does it means? what will be do and how?

Response: Thank you for this helpful comment. Please also see response to comments above. Our transcriptomic methods have been previously published but we believe that specifying these here are

inappropriate, although the relevant references have now been included. The laboratory monitoring assays are not part of the clinical trial protocol.

Page 140 line 41: "Bacterial cultures should be obtained from the site of infection" -- same as before: how many colonies will be tested, how to estimate the genotypical diversity at each site?

Response: Thank you for these comments, however the protocol has been specifically designed to be pragmatic and easily embedded in routine clinical practice (to improve participant recruitment/retention). As such, many "interventions" such as microbiological sampling are in part determined by the treating clinicians and by standard clinical and microbiological practice of the recruitment site. Whole genome sequencing will be performed on all positive cultures obtained after initiation of phage therapy, however the number of colonies sampled or sub-cultured and submitted for sequencing will be determined by standard microbiological practice of the recruitment site. Beyond this, estimates of genotypic diversity will not be attempted.

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Reviewer: 2 Dr. Patrick Jault, Clinique de la Muette

Comments to the Author:

Well designed protocol.

The first clinical trial previously published on the clinical of phages is not cited in your references (JAULT and Coll , 2019, The Lancet infectious diseases).

Response: Thank you for the kind comment and for pointing out this oversight. The study has now been added as a citation demonstrating safety and tolerability of phage therapy, and as one example of very few published clinical trials to date.

## VERSION 2 – REVIEW

<b>REVIEWER</b>	Rémy Froissart Centre National de la Recherche Scientifique (CNRS), MIVEGEC
<b>REVIEW RETURNED</b>	29-Nov-2022
<b>GENERAL COMMENTS</b>	Thanks for your answers. I am pleased with them.